

Loss of Tuberous Sclerosis Complex-2 Function and Activation of Mammalian Target of Rapamycin Signaling in Endometrial Carcinoma

Karen H. Lu,¹ Weiguo Wu,¹ Bhuvanesh Dave,⁴ Brian M. Slomovitz,⁵ Thomas W. Burke,¹ Mark F. Munsell,² Russell R. Broaddus,³ and Cheryl Lyn Walker⁴

Abstract Purpose: The involvement of phosphatase and tensin homologue deleted on chromosome ten (PTEN) in endometrial carcinoma has implicated phosphatidylinositol 3-kinase signaling and mammalian target of rapamycin (mTOR) activation in this disease. Understanding the extent of mTOR involvement and the mechanism responsible for activation is important, as mTOR inhibitors are currently being evaluated in clinical trials for endometrial carcinoma. Although tuberous sclerosis complex 2 (TSC2) is the "gatekeeper" for mTOR activation, little is known about defects in the TSC2 tumor suppressor or signaling pathways that regulate TSC2, such as LKB1/AMP-activated protein kinase, in the development of endometrial carcinoma.

Experimental Design: We determined the frequency of mTOR activation in endometrial carcinoma (primary tumors and cell lines) and investigated PTEN, LKB1, and TSC2 defects as underlying cause(s) of mTOR activation, and determined the ability of rapamycin to reverse these signaling defects in endometrial carcinoma cells.

Results: Activation of mTOR was a consistent feature in endometrial carcinomas and cell lines. In addition to PTEN, loss of TSC2 and LKB1 expression occurred in a significant fraction of primary tumors (13% and 21%, respectively). In tumors that retained TSC2 expression, phosphorylation of tuberlin at S939 was observed with a high frequency, indicating that mTOR repression by TSC2 had been relieved via AKT phosphorylation of this tumor suppressor. In PTEN-null and LKB1-null endometrial carcinoma cell lines with functional inactivation of TSC2, phosphatidylinositol 3-kinase (PI3K) inhibitors wortmannin and LY294002 were able to inhibit AKT and mTOR signaling and reverse TSC2 phosphorylation. In contrast, although rapamycin inhibited mTOR signaling, it did not relieve phosphorylation of TSC2 at S939.

Conclusions: Inactivation of TSC2 via loss of expression or phosphorylation occurred frequently in endometrial carcinoma to activate mTOR signaling. High-frequency mTOR activation supports mTOR as a rational therapeutic target for endometrial carcinoma. However, whereas rapamycin and its analogues may be efficacious at inhibiting mTOR activity, these drugs do not reverse the functional inactivation of TSC2 that occurs in these tumors.

Authors' Affiliations: ¹Department of Gynecologic Oncology, ²Division of Quantitative Sciences, and ³Department of Pathology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas; ⁴Department of Carcinogenesis, The University of Texas M. D. Anderson Cancer Center, Smithville, Texas; and ⁵Division of Gynecologic Oncology, Weill Medical College of Cornell University, New York, New York

Received 2/6/07; revised 6/28/07; accepted 10/26/07.

Grant support: National Cancer Institute Gynecology Specialized Program of Research Excellence grant CA098258 to M. D. Anderson Cancer Center and NIH grants CA63613 (C.L. Walker) and ES07784.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

Requests for reprints: Cheryl Lyn Walker, Department of Carcinogenesis, The University of Texas M. D. Anderson Cancer Center, 1808 Park Road 1C, P.O. Box 389, Smithville, TX 78957. Phone: 512-237-9550; Fax: 512-237-2475; E-mail: cwalker@wotan.mdacc.tmc.edu.

© 2008 American Association for Cancer Research.
doi:10.1158/1078-0432.CCR-07-0321

Endometrial cancer is the most common cancer of the female genital tract, estimated to account for ~40,880 new cancer cases and 7,310 deaths in the United States in 2005 (1). Outcome is heavily influenced by histologic type, initial stage, and grade of tumor, with overall survival ranging from 36% to 95%, depending on the subset of patients analyzed (2). The better-prognosis subgroups include patients with tumor confined to the uterus (stage I) and lower-grade tumors. Conversely, extrauterine disease, serous or clear cell histology, and grade 3 tumors carry a poorer prognosis.

Most endometrial cancers are of the endometrioid histologic subtype, which exhibit loss of function of the tumor suppressor phosphatase and tensin homologue deleted on chromosome ten (*PTEN*) in 30% to 80% of cases (3). *PTEN* is a member of the family of tumor suppressor genes that predispose to the phakomatoses; tumor suppressor gene syndromes including Cowden's disease (*PTEN*), tuberous sclerosis complex (*TSC1* and *TSC2*), and Peutz-Jeghers

syndrome (LKB1). The phakomatoses are characterized by a high propensity to develop preneoplastic or benign lesions with a low probability of malignant transformation (hamartomas), but they also confer a significantly increased risk of adenocarcinomas and other malignant neoplasms at a number of anatomic sites (4). Germ-line defects in *PTEN* result in increased susceptibility to breast and thyroid cancer (5) and *PTEN* defects occur with a high frequency in several sporadic cancers, including gliomas, melanomas, prostate carcinoma, and endometrial carcinoma (6–11). Hereditary *LKB1* defects occurring in Peutz-Jeghers syndrome predispose to gastrointestinal and reproductive tract tumors and breast cancer (12), but *LKB1* defects are rarely observed in sporadic cancers other than lung cancer and melanoma (13–16). Germ-line *TSC2* mutations not only are associated with the development of syndromic hamartomas of various organs, primarily the brain, lung, kidney, and skin, but also confer an increased risk of renal cell carcinoma (17). Although not as well studied as either *PTEN* or *LKB1*, there is little evidence for the involvement of *TSC1* or *TSC2* in the development of sporadic tumors other than a subset of bladder carcinomas (*TSC1*; ref. 18), angiomyolipoma (19), and astrocytomas (20).

The *PTEN*, *LKB1*, and *TSC2* tumor suppressors participate in a common signaling pathway controlling the activity of the mammalian target of rapamycin (mTOR), which regulates protein synthesis and cell growth (Supplementary Fig. S1). *PTEN* functions as a lipid and protein phosphatase, inhibiting the ability of PDK1 to activate AKT (21). Loss of *PTEN* function results in constitutive AKT activation and phosphorylation of downstream targets including *TSC2* (22, 23). The *TSC2* tumor suppressor acts to inhibit mTOR signaling by repressing the small GTPase Rheb, which activates mTOR (23, 24). Phosphorylation of *TSC2* by AKT at S939 and S981 causes it to partition into the cytosol away from its target Rheb and its activation partner *TSC1* in the membrane, relieving repression of Rheb and allowing it to activate mTOR (25). Loss of *PTEN* function therefore results in functional inactivation of the *TSC2* tumor suppressor via AKT phosphorylation.

In addition to regulation by phosphatidylinositol 3-kinase (PI3K)/AKT, *TSC2* is also regulated by AMP-activated protein kinase (AMPK), which, in contrast to AKT, phosphorylates *TSC2* to activate its tumor suppressor function (26, 27). When cellular energy stores are reduced and AMP levels in the cell increase, AMPK becomes activated, phosphorylating and activating *TSC2* to inhibit mTOR signaling and reduce energy-consuming processes such as protein synthesis. AMPK is an immediate downstream target of *LKB1*, and it is phosphorylated and activated by this kinase (28). Thus, *TSC2* acts as a cellular rheostat, coordinating mitogenic signals via PI3K/AKT signaling with the energy status of the cell, via *LKB1*/AMPK signaling (Supplementary Fig. S1). When mitogenic signals and cellular energy levels are high, *TSC2* is inactive, permitting mTOR signaling and protein synthesis. When mitogenic signals and cellular energy levels are low, *TSC2* is active, repressing mTOR signaling and inhibiting protein synthesis.

We report here that a high level of mTOR activity is a consistent feature of endometrial carcinoma. Activation of mTOR occurred in tumors that had lost *PTEN*, but loss of *LKB1* and *TSC2* was also frequently observed in primary tumors, as was functional inactivation of *TSC2* via phosphorylation at S939. Furthermore, inhibition of mTOR with rapamycin in

endometrial carcinoma cell lines representative of *PTEN*- and *LKB1*-null tumors was effective at reducing mTOR signaling. However, phosphorylation (and functional inactivation) of *TSC2* was not affected by rapamycin in these cells. Thus, inactivation of *TSC2* via multiple mechanisms and subsequent activation of mTOR signaling is a common event in endometrial carcinoma, and whereas rapamycin is efficacious at repressing mTOR signaling, this inhibitor is unable to reverse the functional inactivation of *TSC2* that also occurs in these tumors.

Materials and Methods

Cells and culture conditions. AN3CA, Hec1A, and Hec1B cell lines were obtained from American Type Culture Collection and were maintained in MEM (AN3CA) or McCoy's (Hec1A and Hec1B) supplemented with 10% fetal bovine serum. The Ishikawa cell line was obtained from Dr. Marco Gottardis (Bristol-Myers Squibb, Princeton, NJ) and was maintained in DMEM/F-12 (1:1 ratio) plus 10% fetal bovine serum. RL95-2 (CRL-1671) cell line, purchased from American Type Culture Collection, was maintained in DMEM/F-12 supplemented with 10% fetal bovine serum. All tissue culture growth media were from Invitrogen and the fetal bovine serum from Hyclone. Cells were cultured at 37°C, 5% CO₂, and 95% humidity. For serum stimulation, cells were grown to 70% confluence and serum starved for 24 h, then appropriate cells were stimulated in media supplemented with 20% fetal bovine serum for 1 h. For inhibitor experiments, cells were grown to 70% confluence and serum starved for 24 h before adding the inhibitor. LY294002 (Sigma), wortmanin (Sigma), and rapamycin (Sigma) were applied at 20 μmol/L, 100 nmol/L, and 200 nmol/L, respectively, and vehicle (DMSO) to AN3CA, Hec1A, and Ishikawa cells as indicated.

Protein isolation and Western blot analysis. Cells were grown to 70% confluence in 15-cm culture dishes for protein isolation. Plates were washed twice with ice-cold PBS and scraped with 200 μL of 1× lysis buffer [Cell Signaling Technology; 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na₃VO₄, 1× Roche complete protease inhibitor]. Cells were mechanically lysed by sonication and pelleted by centrifugation at 14,000 rpm at 4°C for 10 min. The supernatant was collected and stored at -80°C for Western blot analysis. Tumor tissues were flash frozen and stored at -80°C. To prepare lysates, a small portion (0.5 cm³) of tumor was isolated and crushed with a small mortar and pestle over dry ice and the resulting pulverized tissues were collected with liquid nitrogen and transferred into a microcentrifuge tube. Radioimmunoprecipitation assay buffer (0.5–1.0 mL; 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 0.15 mol/L NaCl, 0.01 mol/L NaPO₄, 2 mmol/L EDTA, 200 mmol/L phenylmethylsulfonyl fluoride, 100 mmol/L activated sodium orthovanadate, and 1× Roche complete protease inhibitor) was added and samples were rotated at 4°C for 2 h. After rotations, the samples were centrifuged at 14,000 rpm at 4°C for 10 min. The resulting supernatant was collected, aliquoted, and stored at -80°C for future analysis. Lysate protein concentrations were determined with BCA Protein Assay Kit (Pierce Biotechnology).

Samples of protein from each sample (30 μg) were size separated by SDS-PAGE and transferred overnight at 4°C onto polyvinylidene difluoride membranes (Pierce Biotechnology). Membranes were blocked in a TBST (TBS plus 0.05% Tween 20) + 5% nonfat milk solution for 1 h. Membranes were incubated in a primary antibody solution for 2 h at room temperature with varying antibody (1:500–1:2,000) and milk (3–5%) concentrations. All primary antibodies were purchased from Cell Signaling Technology. The first *LKB1* antibody, purchased from Calbiochem, was replaced with a second *LKB1* antibody (Cell Signaling) and used in subsequent analysis. Membranes were then washed thrice with TBST solution for 10 min each and then

incubated for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) at room temperature. Membranes were washed with the same procedure as previously mentioned and visualized with LumiGLO chemiluminescent reagents (Kirkegaard and Perry Laboratories) or the ECL+ kit (Amersham Pharmacia Biotech) for more sensitive detection. Membrane immunoreactivity was detected with X-ray film (BioMax, Eastman Kodak).

Immunohistochemistry. Formalin-fixed, paraffin-embedded endometrioid endometrial carcinoma (EEC) specimens from patients surgically treated at The University of Texas M. D. Anderson Cancer Center were obtained from a study protocol approved by the Committee for the Protection of Human Subjects. The immunohistochemical staining for PTEN, p-AKT, and p-S6K was done on 5- μ m sections of formalin-fixed, paraffin-embedded EEC specimens with DAKO LASB kits (DakoCytomation). Tissue sections were incubated at 60°C for 1 h, deparaffinized in xylene, and hydrated in a graded series of ethanol solutions (from 100% to 80%). After a rinse in PBS composed of 137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L KH_2PO_4 , and 10 mmol/L Na_2HPO_4 (pH 7.4), endogenous peroxidase activity was quenched by incubating the sections for 15 min with 0.3% H_2O_2 in absolute methanol. After a 10-min rehydration in PBS, the sections were heated in a microwave oven for 3 min in 10 mmol/L citrate buffer for antigen retrieval. After incubation with blocking serum (4% normal horse serum) for 30 min at room temperature, the sections were incubated overnight at 4°C with monoclonal antibodies against human PTEN (1:200; Cascade Bioscience, Inc.), p-AKT (Ser⁴⁷³) (1:200; Cell Signaling Technology), and p-S6K (Thr³⁸⁹) (1:200; Cell Signaling Technology). Sections of human breast cancer tissue were used as positive controls for these antibodies, and EEC sections incubated without antibodies were used as negative controls. After a PBS rinse, the sections were treated with a biotinylated goat antimouse antibody for 15 min. The sections were washed with PBS thrice and then incubated with an avidin-biotinylated horseradish peroxidase macromolecular complex for 10 min, according to the manufacturer's instructions for the LASB kit. The peroxidase was visualized by incubating the sections with diaminobenzidine for 10 min. The sections were counterstained with hematoxylin, dehydrated in a series of ethanol solutions, and cleared with xylene; coverslips were then placed over the sections for evaluation by light microscopy.

Scoring for the expression of PTEN was done as previously described (29). PTEN scores were assigned semiquantitatively according to the percentage of cells stained (1, <25%; 2, 25-50%; 3, >50%) and the intensity of staining (1, weak; 2, moderate; 3, strong). The two scores were then multiplied. When <25% of the cells were stained or the intensity of staining was weak, the product of the two scores was ≤ 3 , and expression was categorized as "low" expression. When at least 25% of the cells were stained and the intensity of staining was moderate or strong, the product of the two scores was at least 4, and the expression was categorized as "high" expression. Negative or "absent" staining was defined as <5% of cells showing staining.

Statistics. The associations of PTEN expression, AKT activation, and S6 phosphorylation with tumor grade were assessed with the χ^2 test.

The association between S6 phosphorylation and grade, stratified by PTEN status, was assessed by the Cochran-Mantel-Haenszel test. The Cochran-Mantel-Haenszel test was also used to assess the association between S6 phosphorylation and grade, stratified by AKT activation. $P < 0.05$ was considered statistically significant. Cohen's κ statistic was used to evaluate the association between tuberin expression and LKB1 expression and the association between tuberin phosphorylation at S939 and activation of AKT. A κ statistic between 0.40 and 0.70 was considered to represent fair to good agreement beyond chance.

Results

Loss of TSC2 and LKB1 is common in endometrial carcinoma. In a series of 75 EECs examined, immunohistochemistry revealed that PTEN expression was reduced or absent in 44% of primary tumors (Supplementary Table S1), consistent with data in the literature for PTEN involvement in this disease. These tumors were further examined by immunohistochemistry for expression of phospho-AKT and phospho-S6, markers of AKT and mTOR activation, respectively. As shown in Supplementary Table S1, the majority of tumors exhibited AKT and mTOR activation. AKT activation correlated significantly with activation of mTOR signaling as assessed by phosphorylation of S6 ($P \leq 0.02$; Table 1). However, a significant portion of tumors had detectable PTEN, and in some PTEN-expressing tumors in which AKT was not activated, activation of mTOR signaling was evident (Table 1). As shown in Supplementary Table S2, there was no significant difference in S6 phosphorylation between PTEN(+) and PTEN(-) tumors ($P = 0.793$), suggesting that deregulation of mTOR signaling was occurring via other mechanisms (Supplementary Table S2 and Supplementary Fig. S2). The association between S6 phosphorylation and grade, stratified by PTEN activation, was not statistically significant ($P = 0.2249$). There was no association between S6 phosphorylation and tumor grade in the PTEN(-) tumors ($P = 0.5369$); neither did there seem to be an association with S6 phosphorylation and grade in the PTEN(+) tumors ($P = 0.4352$).

To determine if tumor suppressors other than PTEN that regulate mTOR signaling were potentially involved in these tumors, we evaluated the expression of TSC2 and LKB1 in a subset of tumors for which frozen material was available for Western blot analysis (available antibodies for TSC2 and LKB1 are unsuitable for immunohistochemistry). Initially, 38 primary tumors were examined by Western blot analysis for expression of TSC2 (the protein product of which is tuberin) and LKB1 (Fig. 1A and B). Of these 5 of 38 (13%) were negative for tuberin and 8 of 38 (21%) were negative for LKB1. As shown in Table 2, this analysis was repeated on a

Table 1. Correlation between activation of S6 and activation of AKT in endometrioid endometrial cancers

Histologic type	Total no.	Phosphorylation of S6 in EEC with AKT activation, n (%)	Phosphorylation of S6 in EEC without AKT activation, n (%)
EEC	75	48/58 (83)*	8/17 (47)
Grade 1	22	14/17 (82)	2/5 (40)
Grade 2	34	19/25 (76)	4/9 (44)
Grade 3	19	15/16 (94)	2/3 (67)

NOTE: Phosphorylation of S6 was significantly associated with AKT activation.

* $P \leq 0.02$.

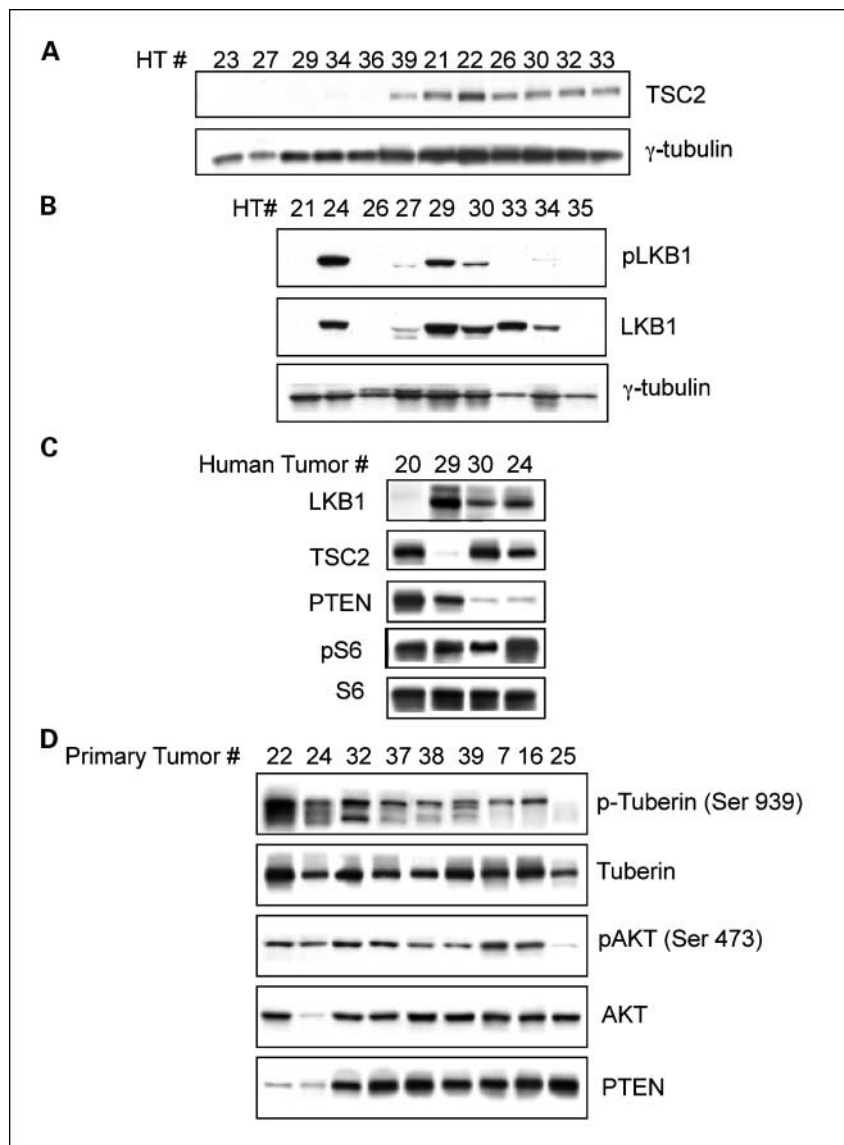


Fig. 1. TSC2, LKB1, and PTEN defects in endometrial carcinomas. Western blot analysis was done to detect TSC2/tuberin (A), LKB1 (B), and PTEN (C) expression. All tumors exhibited activated mTOR as evidenced by phosphorylation of S6 (C). D, primary tumors were examined by Western blot analysis for phosphorylation of tuberin at S939, the AKT phosphorylation site that inactivates this tumor suppressor. Levels of phospho-tuberin correlated with AKT activity (phospho-AKT).

subset of 26 tumors using a second LKB1 antibody, which in our hands was more sensitive than the first (data not shown). Similar data to that obtained with the initial antibody were obtained in this replicate, with 5 of 26 (19%) tumors exhibiting loss of LKB1 expression. Loss of expression was defined as <50% expression relative to normal endometrium.

Table 2. Tuberin and LKB1 expression in primary tumors

Total no. tumors = 26	Tuberin positive	Tuberin negative
LKB1 negative	4	1
LKB1 positive	15	6

NOTE: In general, tumors were either tuberin negative or LKB1 negative, although a single tumor expressed neither LKB1 nor TSC2.

However, in addition to loss of expression, some LKB1-expressing tumors (12 of 26) seemed to have a functional deficiency, as identified by the absence of phosphorylation at S428, which is required for many of the biological functions of LKB1 (Fig. 1B; refs. 30–32). Thus, LKB1 deficiency (loss of expression or absence of phosphorylation) was observed in 17 of 26 (65%) tumors.

Because a significant proportion of tumors had lost tuberin or LKB1 expression, we investigated mTOR activation in these tumors by assessing the extent of S6 phosphorylation. Of the 38 tumors examined, 35 had high levels of phospho-S6 indicative of activated mTOR signaling. As shown in Fig. 1C, S6 phosphorylation was observed in tumors lacking TSC2, LKB1, or PTEN, suggesting that loss of TSC2 expression, constitutive activation of AKT due to loss of PTEN, or an inability to activate AMPK due to loss of LKB1 may all contribute to up-regulation of mTOR signaling. Immunohistochemistry confirmed that PTEN-expressing tumors that were TSC2 or LKB1 null exhibited elevated mTOR activity as assessed by phospho-S6 staining (Supplementary Fig. S2).

Table 3. Correlation of tuberlin phosphorylation with AKT activation

	Tuberlin P-S939 positive	Tuberlin P-S939 negative
AKT (active) P-S473-positive	16	7
AKT (inactive) P-S473-negative	4	11

NOTE: Phosphorylation of tuberlin at S939 was significantly associated with the presence of active AKT (phospho-S473) as determined by Cohen's κ statistic (0.415; 95% confidence interval, 0.127-0.702).

The expression of high levels of phospho-S6, indicative of activated mTOR signaling, observed in tumors that retained tuberlin expression (Fig. 1), suggested that this tumor suppressor might be functionally inactivated in these tumors. To determine whether this was the case, we examined the phosphorylation status of TSC2 at S939, an AKT phosphorylation site that inactivates tuberlin by partitioning it from the membrane to the cytosol (25). Whereas the majority of tumors continued to express tuberlin (33 of 38), as shown in Fig. 1D, this tumor suppressor seemed to be functionally inactive as assessed by phosphorylation on S939, and the presence or absence of S939 phosphorylation was significantly associated with AKT activation in these tumors (Fig. 1D; Table 3). As shown in Fig. 1D, tumors in which TSC2 was phosphorylated at S939 also had activated AKT (as assessed by phosphorylation at S473), whereas in tumors in which tuberlin was expressed but not phosphorylated, AKT phosphorylation was absent or barely detectable. Interestingly, phosphorylation of tuberlin at S939 was observed in both PTEN-null and PTEN-expressing tumors that had activated AKT (Fig. 1D).

Identification of PTEN and LKB1 defects in endometrial carcinoma cell lines. Several endometrial carcinoma-derived cell lines are available that differ in characteristics such as the primary tumor site from which they were derived (uterus versus metastatic lesions) or steroid hormone responsiveness. However, these lines have not been comprehensively evaluated in terms of TSC2, PTEN, or LKB1 tumor suppressor gene expression. To identify cell lines representative of the specific tumor suppressor defects we identified in primary tumors, PTEN, LKB1, and TSC2 expression was characterized in a panel of five endometrial carcinoma cell lines: AN3CA, Ishikawa, Hec1A, Hec1B, and RL95-2. As shown in Fig. 2A, of the five cell lines examined, two were PTEN null (AN3CA and RL95-2) and one was LKB1 null (Ishikawa) whereas all five lines expressed TSC2. AN3CA and Ishikawa were selected for further study as representative of PTEN-null and LKB1-null tumors, respectively.

Analysis of PI3K signaling in these cell lines was consistent with the presence of these tumor suppressor defects, with both AN3CA and Ishikawa exhibiting constitutively active mTOR signaling, as determined by levels of phospho-S6 in the absence of serum (Fig. 3), which were equivalent to Hec1B cells with mutant PIK3CA (ref. 33; data not shown). The constitutively active mTOR signaling observed in AN3CA and Ishikawa cells suggested that TSC2 had been functionally inactivated as a consequence of loss of PTEN, LKB1, and/or activation of AKT in

these two lines. To determine if this was indeed the case, AKT activation and TSC2 phosphorylation on S939 was examined. As shown in Fig. 2B, phospho-AKT was higher in the AN3CA and Ishikawa cell lines relative to Hec1A cells, which express PTEN, LKB1, and TSC2 (Fig. 2A and B). Increased S939 phosphorylation of TSC2 also occurred in both AN3CA and Ishikawa cells relative to Hec1A cells. In Ishikawa cells, phosphorylation of TSC2 at S939 increased dramatically concomitant with the activation of AKT, whereas both AKT and tuberlin were constitutively phosphorylated in AN3CA cells, consistent with the constitutive activation of PI3K in these cells. To further confirm that TSC2 was being phosphorylated, and thus inactivated, in these cells, glutathione S-transferase

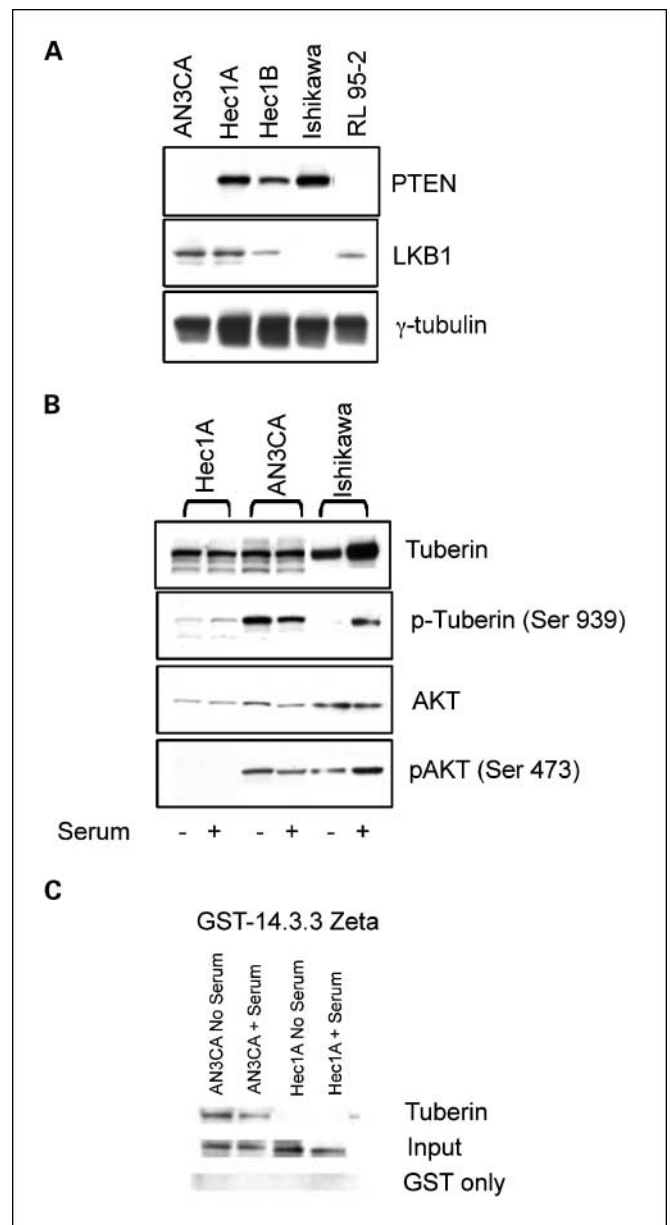


Fig. 2. mTOR signaling in endometrial carcinoma cell lines. *A*, Western blot analysis identified cell lines that had lost PTEN or LKB1 expression. *B*, phosphorylation of tuberlin correlated with activation of AKT in AN3CA and Ishikawa. *C*, GST-14-3-3 recognized phosphorylated tuberlin in AN3CA.

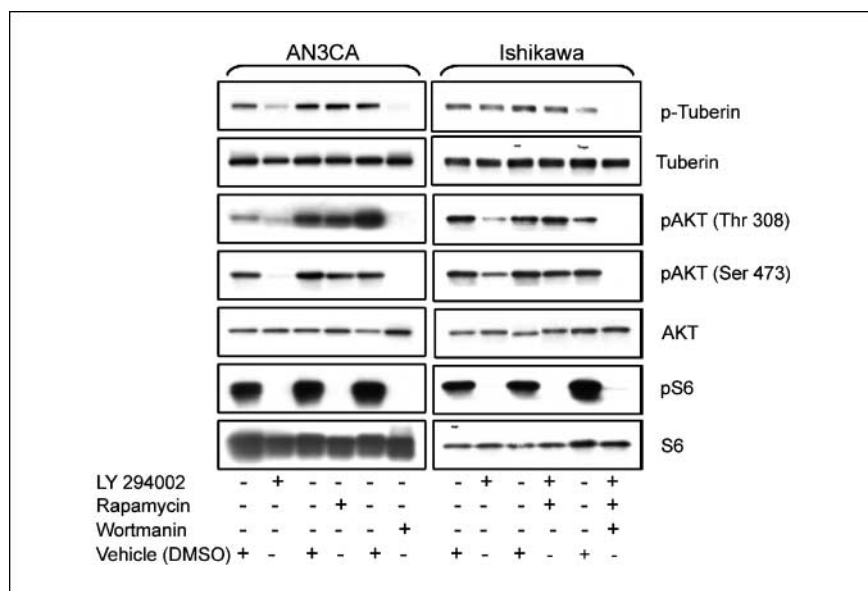


Fig. 3. Inhibition of PI3K and mTOR signaling in endometrial carcinoma cell lines. PI3K inhibitors (LY294002 and wortmannin) inhibit AKT and mTOR activity and reduce levels of phospho-tuberin (S939). Rapamycin inhibits mTOR signaling but does not reduce AKT activity or tuberin phosphorylation. DMSO was used as vehicle control.

(GST)-14-3-3 pull-down experiments were done in AN3CA cells. 14-3-3 proteins bind TSC2 in response to phosphorylation by AKT at S939 and S981 and serve to sequester TSC2 away from its GTPase-activating protein target Rheb and inactivate this tumor suppressor (25). As shown in Fig. 2C, GST-14-3-3 recognized TSC2 from AN3CA cells, which had high phospho-TSC2 levels, whereas TSC2 from Hec1A cells was not recognized by GST-14-3-3, consistent with retention of TSC2 functional activity in these cells.

Effect of PI3K and mTOR inhibitors on PI3K signaling in endometrial carcinoma cells. Activation of mTOR signaling in primary tumors and tumor-derived cell lines with PTEN and LKB1 defects suggested that mTOR would be a viable therapeutic target for endometrial carcinoma. To investigate the ability of mTOR inhibition to block signaling and determine its effect on tuberin activity (i.e., phosphorylation) in endometrial carcinoma cells, we treated AN3CA and Ishikawa cells with mTOR (rapamycin) or PI3K (wortmannin and LY294002) inhibitors and monitored AKT activity, TSC2 phosphorylation, and mTOR signaling. As shown in Fig. 3, rapamycin was effective at reducing mTOR signaling, as assessed by diminished phospho-S6 levels in both PTEN-null AN3CA and LKB1-null Ishikawa cells. Importantly, levels of phospho-AKT remained unchanged in these cells, as did levels of phospho (inactive) tuberin. In contrast to mTOR inhibition, PI3K inhibition by wortmannin or LY294002 diminished both mTOR signaling and TSC2 phosphorylation, concomitant with reduction of AKT activity. Therefore, in contrast to mTOR inhibition by rapamycin, a signaling blockade upstream of AKT is able to abrogate mTOR signaling and reverse the inactivation of TSC2 by AKT phosphorylation.

Discussion

Loss of function of the PTEN tumor suppressor gene occurs in a significant fraction of endometrial carcinomas, resulting in dysregulated PI3K signaling and activation of AKT. Whereas PTEN alterations are well documented in endometrial carcinoma, signaling defects involving the downstream effector mTOR

or other tumor suppressors that regulate mTOR signaling, including TSC2 and LKB1, have not been investigated in these tumors. We found that activation of mTOR signaling was a consistent feature of both the primary tumors and endometrial carcinoma-derived cell lines examined in this study. Expression of TSC2 and its upstream regulators, LKB1 and PTEN, were all lost in a significant number of primary tumors and, in the case of PTEN and LKB1, in endometrial carcinoma cell lines as well, resulting in relief of mTOR repression in endometrial carcinoma cells.

The TSC2 tumor suppressor gene serves as the “gatekeeper” for mTOR signaling. Loss of expression or functional inactivation of this tumor suppressor via PI3K or mitogen-activated protein kinase signaling and phosphorylation by AKT, extracellular signal-regulated kinase, p90RSK, or p38-MK2 (23) relieves tuberin’s repression of its GTPase-activating protein target Rheb to activate mTOR signaling. Activation of mTOR signaling promotes cell growth via ribosome biogenesis and increased protein translation (34). Loss of TSC2 expression or functional inactivation of tuberin via AKT-mediated phosphorylation at S939 occurred in a significant proportion of endometrial carcinomas and tumor-derived cell lines, consistent with the high frequency of mTOR activation in endometrial carcinoma. Data obtained using Ishikawa endometrial carcinoma cells have shown similar functional consequences for inactivation of tuberin (35) as we report here. In these experiments, prostaglandin E₂ promoted growth of Ishikawa cells via activation of AKT and phosphorylation of tuberin. Prostaglandin E₂-mediated growth and phosphorylation of tuberin was reversible by treatment with LY294002 or wortmannin, which inhibited AKT signaling. Thus, inactivation of tuberin to relieve repression of mTOR signaling seems to be a common event in primary endometrial carcinomas and an important target for stimulation of cell growth in these cells.

Due to the frequency with which mTOR is activated in tumors, the mTOR inhibitor rapamycin and its analogues are currently being explored as cancer therapeutics for several types of cancer including endometrial carcinoma (36, 37). Two phase II trials of mTOR inhibitors are currently ongoing for women

with recurrent endometrial cancer, one using CCI-779 and one using the oral agent RAD001. However, recently, it has become clear that PI3K signaling is also regulated by a negative feedback loop downstream of mTOR (22). When activated by mTOR, S6K phosphorylates and inhibits IRS1, which is upstream of PI3K and participates in activation of PI3K via insulin/insulin-like growth factor II receptor signaling (38). As a consequence of loss of IRS1 signaling to PI3K, AKT activity is down-regulated in some tumors that have activated mTOR, including tumors that have lost TSC2 function. This has now been shown to have clinical implications, as the mTOR inhibitor rapamycin and its analogues RAD001 and CCI-771 are efficacious at inhibiting mTOR but can induce AKT activation in tumors via relief of S6K repression of IRS1, with potential adverse consequences for the patient (39). Whereas activation of mTOR signaling seen in endometrial carcinomas and tumor-derived cell lines indicates that mTOR is a viable therapeutic target for this disease, it is also possible that relief of S6K repression of IRS1 and up-regulation of AKT could occur in response to rapamycin. In our study, we found that AKT activity was high in primary tumors and the panel of cell lines examined, and did not increase with rapamycin, suggesting that negative feedback to AKT was not occurring in either tumors or tumor-derived cell lines with PTEN, LKB1, or TSC2 defects, strengthening the rationale for mTOR as a therapeutic target for endometrial carcinoma. Therefore, endometrial carcinoma would seem to be a good candidate for mTOR inhibitors due to the high frequency of mTOR activation that occurs in these tumors and the absence of negative feedback of AKT signaling by S6K.

In addition to activation of mTOR signaling, loss of tuberin function has other adverse consequences for the cell. Tuberin has been implicated in regulation of nuclear hormone receptor function (40), activity of the cyclin-dependent kinase inhibitor p27 (41), Wnt/ β -catenin and nuclear factor- κ B signaling (42–44), and regulation of the cytoskeleton to modulate cell motility and endocytosis (45, 46). Targeting of mTOR is unlikely to reverse the functional inactivation of tuberin that occurs in a large number of these tumors, and would therefore not inhibit or reverse other defects that occur as a result of loss of function of this tumor suppressor gene in endometrial carcinoma. Drugs that modulate TSC2 activity, directly or via activation of AMPK, could have clinical advantages over rapamycin analogues, as they could potentially reverse the mTOR signaling defect and also restore TSC2 function.

Phosphorylation at a conserved serine residue of LKB1 by protein kinase A has been shown to be important for various biological processes such as anterior-posterior axis formation in *Drosophila* (31), early signaling for axon initiation in neuronal development (32), and LKB1-dependent neuronal polarization (30). All these processes depend on activation of LKB1 signaling to AMPK. In our panel of tumors, we observed that the presence of LKB1 may not necessarily be sufficient for functionality of this signaling pathway. Owing to lack of phosphorylation could contribute to loss of function of LKB1/AMPK signaling to TSC2, thereby contributing to elevated mTOR signaling. Because this possibility has clinical implications, it warrants further investigation.

Although this is the first study to characterize LKB1 and TSC2 expression in endometrial carcinoma tumors and cell lines, PTEN expression in several endometrial carcinoma cell lines has previously been reported. However, data on expression of PTEN

in the available endometrial carcinoma lines are not always consistent, likely due to differences in the source of the cells. AN3CA and RL95-2 cells, in which we observed PTEN expression to be undetectable, have previously been reported to have frameshift mutations, as do Ishikawa cells which continue to express PTEN, whereas the PTEN sequence is normal in Hec and KLE cells (47, 48). In those reports and our study, AN3CA and Hec cell lines were obtained from American Type Culture Collection whereas Ishikawa cells were obtained from other sources. However, others have reported that Ishikawa and Hec1B cells do not express PTEN whereas AN3CA do (49, 50), although the source of the cells used in that study was not reported. Thus, caution must be exercised when using these (or other) cell lines, and it is advisable to characterize each strain of cells before use.

Cowden's disease caused by germ-line defects in *PTEN* is associated with increased risk of endometrial carcinoma (5), whereas neither Peutz-Jeghers (12) nor tuberous sclerosis complex (17), caused by germ-line defects in *LKB1* and *TSC2*, respectively, have been noted to have a significantly increased incidence of endometrial carcinoma. However, Peutz-Jeghers patients do have a 2-fold elevated risk for development of gynecologic lesions (endometrial carcinoma is included in this category; ref. 12), and although endometrial carcinoma is quite rare in women under the age of 40 years, there is a case report of invasive endometrial carcinoma arising in a patient with tuberous sclerosis (51). Importantly, the absence of an association of a particular tumor type with a hereditary cancer syndrome does not preclude the participation of tumor suppressor genes responsible for that syndrome in sporadic tumors. This is evident for prostate carcinoma, which does not occur with an elevated frequency in patients with Cowden's disease but nevertheless a high frequency of PTEN defects (70–80% of primary tumors) is observed in sporadic prostate carcinoma (52). Similarly, bladder cancer, which does not occur with an increased frequency in tuberous sclerosis patients, has been reported to have TSC1 defects in a significant proportion of sporadic tumors (18).

Whereas type I endometrial carcinomas (endometrioid tumors) are associated with defects in PTEN and have a better prognosis than type II tumors (serous and clear cell carcinomas), differences may exist between outcomes for different subsets of endometrioid type I tumors. Due to the relatively small number of tumors analyzed in this study, it was not possible to perform a statistical correlation between TSC2 or LKB1 status and tumor grade. However, whereas loss of LKB1 expression was observed in grade 1, 2, and 3 endometrioid tumors, loss of TSC2 expression was only observed in grade 1 and 2 tumors. This trend is potentially interesting, as PTEN-positive and phosphorylated-AKT-negative tumors, which would be predicted to have alterations in TSC2 or LKB1, have a better prognosis than PTEN-negative tumors with activated AKT (53, 54). Thus, future studies to stratify endometrioid tumors by PTEN, TSC2, and LKB1 status are warranted to determine if loss of specific tumor suppressor gene(s) correlates with outcome and, ultimately, response to targeted therapies.

Acknowledgments

We thank Drs. Diego Castrillon and Marsha Frazier for critical review of the manuscript, Sarah Henninger for manuscript preparation, and Jennifer Widener and Melisa Portis for excellent technical assistance.

References

1. Jemal A, Murray T, Ward E, et al. Cancer statistics, 2005. *CA Cancer J Clin* 2005;55:10–30.
2. Burke T, Eifel PJ, Muggia FM. Cancer of the uterine body. In: DeVita VT, Jr., Hellman S, Rosenberg SA, editors. *Cancer: principles & practice of oncology*. Philadelphia: Lippincott; 1997. p. 1478–99.
3. Latta E, Chapman WB. PTEN mutations and evolving concepts in endometrial neoplasia. *Curr Opin Obstet Gynecol* 2002;14:59–65.
4. Knudson AG. Chasing the cancer demon. *Annu Rev Genet* 2000;34:1–19.
5. Pilarski R, Eng C. Will the real Cowden syndrome please stand up (again)? Expanding mutational and clinical spectra of the PTEN hamartoma tumour syndrome. *J Med Genet* 2004;41:323–6.
6. Rasheed BK, Stenzel TT, McLendon RE, et al. PTEN gene mutations are seen in high-grade but not in low-grade gliomas. *Cancer Res* 1997;57:4187–90.
7. Wang SI, Puc J, Li J, et al. Somatic mutations of PTEN in glioblastoma multiforme. *Cancer Res* 1997;57:4183–6.
8. Liu W, James CD, Frederick L, Alderete BE, Jenkins RB. PTEN/MMAC1 mutations and EGFR amplification in glioblastomas. *Cancer Res* 1997;57:5254–7.
9. Guldberg P, thor Straten P, Birck A, Ahrenkiel V, Kirkin AF, Zeuthen J. Disruption of the MMAC1/PTEN gene by deletion or mutation is a frequent event in malignant melanoma. *Cancer Res* 1997;57:3660–3.
10. Tsao H, Zhang X, Benoit E, Haluska FG. Identification of PTEN/MMAC1 alterations in uncultured melanomas and melanoma cell lines. *Oncogene* 1998;16:3397–402.
11. Dahia PL, Marsh DJ, Zheng Z, et al. Somatic deletions and mutations in the Cowden disease gene, PTEN, in sporadic thyroid tumors. *Cancer Res* 1997;57:4710–3.
12. Hearle N, Schumacher V, Menko FH, et al. Frequency and spectrum of cancers in the Peutz-Jeghers syndrome. *Clin Cancer Res* 2006;12:3209–15.
13. Sanchez-Cespedes M, Parrella P, Esteller M, et al. Inactivation of LKB1/STK11 is a common event in adenocarcinomas of the lung. *Cancer Res* 2002;62:3659–62.
14. Avizienyte E, Loukola A, Roth S, et al. LKB1 somatic mutations in sporadic tumors. *Am J Pathol* 1999;154:677–81.
15. Rowan A, Bataille V, MacKie R, et al. Somatic mutations in the Peutz-Jeghers (LKB1/STK11) gene in sporadic malignant melanomas. *J Invest Dermatol* 1999;112:509–11.
16. Guldberg P, thor Straten P, Ahrenkiel V, Seremet T, Kirkin AF, Zeuthen J. Somatic mutation of the Peutz-Jeghers syndrome gene, LKB1/STK11, in malignant melanoma. *Oncogene* 1999;18:1777–80.
17. Gomez MR, Sampson JR, Whittemore VH, editors. *Tuberous sclerosis complex*. 3rd ed. Oxford: Oxford University Press; 1999.
18. Mhawech-Fauceglia P, Cheney RT, Schwaller J. Genetic alterations in urothelial bladder carcinoma: an updated review. *Cancer* 2006;106:1205–16.
19. Henske EP, Neumann HP, Scheithauer BW, Herbst EW, Short MP, Kwiatkowski DJ. Loss of heterozygosity in the tuberous sclerosis (TSC2) region of chromosome band 16p13 occurs in sporadic as well as TSC-associated renal angiomyolipomas. *Genes Chromosomes Cancer* 1995;13:295–8.
20. Wienecke R, Guha A, Maize JC, Jr., Heideman RL, DeClue JE, Gutmann DH. Reduced TSC2 RNA and protein in sporadic astrocytomas and ependymomas. *Ann Neurol* 1997;42:230–5.
21. Cully M, You H, Levine AJ, Mak TW. Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. *Nat Rev Cancer* 2006;6:184–92.
22. Hay N. The Akt-mTOR tango and its relevance to cancer. *Cancer Cell* 2005;8:179–83.
23. Inoki K, Corradetti MN, Guan KL. Dysregulation of the TSC-mTOR pathway in human disease. *Nat Genet* 2005;37:19–24.
24. Li Y, Corradetti MN, Inoki K, Guan KL. TSC2: filling the GAP in the mTOR signaling pathway. *Trends Biochem Sci* 2004;29:32–8.
25. Cai SL, Tee AR, Short JD, et al. Activity of TSC2 is inhibited by AKT-mediated phosphorylation and membrane partitioning. *J Cell Biol* 2006;173:279–89.
26. Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. *Cell* 2003;115:577–90.
27. Corradetti MN, Inoki K, Bardeesy N, DePinho RA, Guan KL. Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome. *Genes Dev* 2004;18:1533–8.
28. Hardie DG. New roles for the LKB1-AMPK pathway. *Curr Opin Cell Biol* 2005;17:167–73.
29. Wu W, Slomovitz BM, Soliman PT, et al. Correlation of cyclin D1 and cyclin D3 overexpression with the loss of PTEN expression in endometrial carcinoma. *Int J Gynecol Cancer* 2006;16:1668–72.
30. Barnes AP, Lilley BN, Pan YA, et al. LKB1 and SAD kinases define a pathway required for the polarization of cortical neurons. *Cell* 2007;129:549–63.
31. Martin SG, St Johnston D. A role for Drosophila LKB1 in anterior-posterior axis formation and epithelial polarity. *Nature* 2003;421:379–84.
32. Shelly M, Cancedda L, Heilshorn S, Sumbre G, Poo MM. LKB1/STRAD promotes axon initiation during neuronal polarization. *Cell* 2007;129:565–77.
33. Oda K, Stokoe D, Taketani Y, McCormick F. High frequency of coexistent mutations of PIK3CA and PTEN genes in endometrial carcinoma. *Cancer Res* 2005;65:10669–73.
34. Thomas GV. mTOR and cancer: reason for dancing at the crossroads? *Curr Opin Genet Dev* 2006;16:78–84.
35. Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* 1995;19:183–232.
36. Granville CA, Memmott RM, Gills JJ, Dennis PA. Handicapping the race to develop inhibitors of the phosphoinositide 3-kinase/Akt/mammalian target of rapamycin pathway. *Clin Cancer Res* 2006;12:679–89.
37. Rowinsky EK. Targeting the molecular target of rapamycin (mTOR). *Curr Opin Oncol* 2004;16:564–75.
38. Manning BD. Balancing Akt with S6K: implications for both metabolic diseases and tumorigenesis. *J Cell Biol* 2004;167:399–403.
39. O'Reilly KE, Rojo F, She QB, et al. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer Res* 2006;66:1500–8.
40. York B, Lou D, Panettieri RA, Jr., Krymskaya VP, Vanaman TC, Noonan DJ. Cross-talk between tuberlin, calmodulin, and estrogen signaling pathways. *FASEB J* 2005;19:1202–4.
41. Rosner M, Freilinger A, Hengstschlager M. The tuberous sclerosis genes and regulation of the cyclin-dependent kinase inhibitor p27. *Mutat Res* 2006;613:10–6.
42. Ghosh S, Tergaonkar V, Rothlin CV, et al. Essential role of tuberous sclerosis genes TSC1 and TSC2 in NF- κ B activation and cell survival. *Cancer Cell* 2006;10:215–26.
43. Inoki K, Ouyang H, Zhu T, et al. TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. *Cell* 2006;126:955–68.
44. Mak BC, Takamaru K, Kenerson HL, Moon RT, Yeung RS. The tuberlin-hamartin complex negatively regulates β -catenin signaling activity. *J Biol Chem* 2003;278:5947–51.
45. Goncharova E, Goncharov D, Noonan D, Krymskaya VP. TSC2 modulates actin cytoskeleton and focal adhesion through TSC1-binding domain and the Rac1 GTPase. *J Cell Biol* 2004;167:1171–82.
46. Astrinidis A, Cash TP, Hunter DS, Walker CL, Chernoff J, Henske EP. The tuberous sclerosis complex 2 tumor suppressor gene regulates Rho activation, cell adhesion, and motility. *Oncogene* 2002;21:8470–6.
47. Matsushima-Nishiu M, Unoki M, Ono K, et al. Growth and gene expression profile analyses of endometrial cancer cells expressing exogenous PTEN. *Cancer Res* 2001;61:3741–9.
48. Yaginuma Y, Yamashita T, Ishiya T, et al. Abnormal structure and expression of PTEN/MMAC1 gene in human uterine cancers. *Mol Carcinog* 2000;27:110–6.
49. Wan X, Yokoyama Y, Shinohara A, Takahashi Y, Tamaya T. PTEN augments staurosporine-induced apoptosis in PTEN-null Ishikawa cells by down-regulating PI3K/Akt signaling pathway. *Cell Death Differ* 2002;9:414–20.
50. Zhou C, Gehrig PA, Whang YE, Boggess JF. Rapamycin inhibits telomerase activity by decreasing the hTERT mRNA level in endometrial cancer cells. *Mol Cancer Ther* 2003;2:789–95.
51. Jaffe JS, Chambers JT. Endometrial adenocarcinoma presenting in a premenopausal patient with tuberous sclerosis. *J Intellect Disabil Res* 2005;49:463–5.
52. Majumder PK, Sellers WR. Akt-regulated pathways in prostate cancer. *Oncogene* 2005;24:7465–74.
53. Uegaki K, Kanamori Y, Kigawa J, et al. PTEN-positive and phosphorylated-Akt-negative expression is a predictor of survival for patients with advanced endometrial carcinoma. *Oncol Rep* 2005;14:389–92.
54. Terakawa N, Kanamori Y, Yoshida S. Loss of PTEN expression followed by Akt phosphorylation is a poor prognostic factor for patients with endometrial cancer. *Endocr Relat Cancer* 2003;10:203–8.

Clinical Cancer Research

Loss of Tuberous Sclerosis Complex-2 Function and Activation of Mammalian Target of Rapamycin Signaling in Endometrial Carcinoma

Karen H. Lu, Weiguo Wu, Bhuvanesh Dave, et al.

Clin Cancer Res 2008;14:2543-2550.

Updated version	Access the most recent version of this article at: http://clincancerres.aacrjournals.org/content/14/9/2543
Supplementary Material	Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2008/07/29/14.9.2543.DC1

Cited articles	This article cites 52 articles, 19 of which you can access for free at: http://clincancerres.aacrjournals.org/content/14/9/2543.full#ref-list-1
Citing articles	This article has been cited by 9 HighWire-hosted articles. Access the articles at: http://clincancerres.aacrjournals.org/content/14/9/2543.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org .
Permissions	To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/14/9/2543 . Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.